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Microbiota in Waterlogged Archaeological Wood: Use of Next-Generation Sequencing to Evaluate the Risk of Biodegradation

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Abstract: Waterlogged archaeological wood (WAW) is considered a precious material, first-hand account of past civilizations. Like any organic material, it is subjected to biodegradative action of microorganisms whose activity could be particularly fast and dangerous during the phases of excavation, storage and restoration. The present work aimed to characterize the microorganisms present in WAW during these tricky periods to evaluate the biological risk it is exposed to. The bacterial and fungal communities inhabiting woods coming from two archaeological sites (Pisa and Naples) were investigated through Next-Generation Sequencing (NGS). High-throughput sequencing of extracted DNA fragments was performed using the reversible terminator-based sequencing chemistry with the Illumina MiSeq platform. The analyses revealed that the two archaeological sites showed distinct richness and biodiversity, as expected. In all the WAWs, the bacterial community harbored mainly Proteobacteria, whereas Bacteroidetes was well represented only in Naples communities and taxa belonging to the phyla Chloroflexi only in the Pisa site. Concerning the fungal community, the two sites were dominated by different phyla: Ascomycota for Naples samples and Basidiomycota for Pisa. Interestingly, most of the identified bacterial and fungal taxa have cellulolytic or ligninolytic ability. These results provide new and useful background information concerning the composition of WAW microbiota and the threat it represents for this precious material.

Keywords: High-throughput sequencing; NGS; 16S rRNAs; ITS2-rDNA sub-region; wood decay bacteria; wood decay fungi; archaeological wood biodegradation; WAW; shipwreck

1. Introduction

Archaeological wood is defined as “wood, used by an extinct human culture, that may or may not have been modified for or by use, and that was discarded by intent or accident into a specific

natural environment” [1]. Waterlogged archaeological wood (WAW) is characterized by a water filled structure and is recovered from water-saturated environments like sites submerged in lake, sea, river or wetland, or land waterlogged sites [2]. After recovery from the site and before museum display, any waterlogged archaeological wooden object must undergo restoration treatments aimed at consolidating the fragile material avoiding excessive dimensional changes or the wood collapse [3,4]. Depending on several factors like object dimension, wood level of degradation or availability of funds, the restoration operations and the phase preceding them may last for several months or even years. Like any organic material, wood is decomposed by biological processes in nature. In waterlogged sites where oxygen is limited, wood biodeterioration is mainly attributed to bacteria. These microorganisms are early colonizers of wood in wet environments, able to attack wood cell walls and pit membranes producing typical degradation patterns (e.g., erosion, tunneling, and cavitation) [5,6]. As long as wood is kept waterlogged, anaerobic or almost anaerobic conditions are maintained and the degradation processes are very slow. During excavation, storage phase, and restoration treatments, the oxygen level to which wooden artifacts are exposed rise and they can be subjected to faster decay forms attributed to fungi. Wood-degrading fungi cause three main types of decay: white, brown and soft rot. White and soft rot, caused respectively by basidiomycetes and ascomycetes/deuteromycetes, are the principal threat for waterlogged wood. By attacking cell wall polysaccharides and lignin, these fungi can lead to a more or less total destruction of wood in a relatively short time span [5–10].

The colonization of WAW by bacteria and fungi has been widely studied to understand the degradation processes affecting this precious material [8,9,11–13], but only little attention has been paid to the biological risk the wood is exposed to during excavation, storage, and/or restoration phases [7,10,14,15]. The study of wood biodeteriogens and their degradative activity usually involves cultural and microscopic analyses [11,16–21]. Over the last decades, molecular techniques have been applied to this field of study. They were mainly based on the amplification and sequencing of DNA extracted from cultured microorganisms and, only in few cases, directly from wood [14,22–26]. Next-generation sequencing (NGS) is an innovative technique that was applied to the study of WAW conservation only in the last couple of years. Although five studies regarding the high-throughput sequencing of microbial communities involved in the biodeterioration of WAW were published [27–31], only two aimed to characterize the microorganisms present inside the wood. The other works focused on the analysis of the storage water and the burial environment or mainly aimed to characterize ancient DNA (aDNA) in order to perform evolutionary studies.

The present work studied, through NGS, the bacterial and fungal communities present in WAW samples during the storage phase preceding the restoration to highlight the biological risk the wood is exposed to during this tricky period. The study involved different wood species coming from two archaeological land sites, the ancient port of Naples and San Rossore in Pisa, and preserved in different conditions after recovery. Previous studies carried out by the Istituto Centrale per il Restauro (ICR, Rome) through traditional microbial cultures and biochemical assays during the excavation and in the following recovery and storage stages, had highlighted the microbial contamination of wood surfaces and of the storage water suggesting the possibility of the biological degradation of wood [15, unpublished data]. The aim of the present study was to evaluate the biological contamination of wood after many years following recovery. The results showed that all wood samples, from both archaeological sites, were exposed to a high biological risk because of the presence of a large number of bacteria and fungi able to degrade cellulose, lignin or their derivatives.

2. Materials and Methods

NGS has been rarely applied to the study of the microbiota present in WAW. Due to the novelty of the application, two protocols for the extraction of microbial DNA from wood were used.

The extraction of microbial DNA from wood was carried out on 10 samples of WAW selected from the material available at the biology laboratory of ICR. The sample name, wood species and provenance are summarized in Table 1.

Table 1. List of waterlogged archaeological wood samples used for the tests.

Sample name	Wood Species	Provenance
N1	Silver fir— <i>Abies alba</i> Mill.	Ancient port of Neapolis—archaeological site of Piazza Municipio, Naples, Italy
N4	Silver fir— <i>Abies alba</i> Mill.	
N9	Elm— <i>Ulmus</i> sp.	
N39	Elm— <i>Ulmus</i> sp.	
N88	Silver fir— <i>Abies alba</i> Mill.	
PF	Ash— <i>Fraxinus</i> sp.	Archaeological site of San Rossore, Pisa, Italy
PO	Elm— <i>Ulmus</i> sp.	
PQ	Oak— <i>Quercus</i> sp.	
PL1	Holm oak— <i>Quercus ilex</i> L.	
PL2	Holm oak— <i>Quercus ilex</i> L.	

Wood remains from Naples were recovered in 2015 and were stored in water at 4 °C in the dark. They pertain the shipwreck named F and are dated back to the end of the II century AD [32]. The samples from Pisa were excavated in 2000 and were stored in water at room temperature. The fragments were sampled from different wooden remains pertaining the urban harbor of Etruscan and Roman Pisa (VII century BC to V century AD) [33–35]. The samples from both sites had never been treated with a biocide. When the analyses were performed, the storage water appeared more or less turbid, depending on the samples, and no biological colonization was perceivable by naked eye in the water or on the wood surface.

In 2008, the wooden remains from Pisa had been characterized and the wood level of degradation had been determined in the frame of a thesis project of the ICR restoration school [36]. In order to evaluate the present state of wood preservation with a quantitative method, to compare it to results obtained in the 2008 situation, and to establish a correlation between the wood degradation and the microbiota composition, analyses for the physical characterization of wood were performed on the Pisa samples selected for the present work. In particular, the maximum water content (MWC) and basic density (D_{bd}) were measured, and the lost wood substance (LWS) was calculated according to the Italian standard and to the most followed protocols [37–41].

The decrease of physical properties accounts for biodegradation processes which occur both during the lying in the waterlogged sites and after the recovery, and the two contributions cannot be distinguished. As no data concerning the state of preservation of Naples remains before the beginning of the present work were available, a certain correlation between the degradation of wood occurred during the storage phase and the present composition of the microbiota could not have been established. Therefore, no physical analyses on these samples are reported here.

The protocols for the DNA extraction were developed basing on the extraction kits Maxwell®RSC Plant DNA Kit (AS1490) (Promega Corporation, Madison, WI, USA) and Maxwell®RSC PureFood GMO and Authentication Kit (AS1600) (Promega Corporation, Madison, WI, USA). In order to compare the extraction efficiency on the same microbial community, both protocols were applied to the same wood sample (Table 2). The only exception is represented by samples PL1 and PL2, for which the wood material was not sufficient to perform a double extraction.

DNA was extracted from 100 mg of wood frozen in liquid nitrogen and homogenized using mortar and pestle. In both protocols, every sample was incubated 5 minutes at 95 °C in a lysis buffer (A509C) (Promega Corporation, Madison, WI, USA) modified with 3% of PVPP. Then, proteinase K (MC5005) (Promega Corporation, Madison, WI, USA) was added in the buffer and the samples were incubated 25 minutes at 65 °C. Finally, after a 13,000-rpm centrifugation, the samples were processed by the two different kits and total genomic microbial DNA was extracted with Maxwell®RSC Instrument (Promega Corporation, Madison, WI, USA)

Table 2. List of samples used for the two DNA extraction protocols.

Protocol 1—Maxwell®RSC Plant DNA Kit	Protocol 2—Maxwell®RSC PureFood GMO and Authentication Kit
1N1	2N1
1N4	2N4
1N9	2N9
1N39	2N39
1N88	2N88
1PF	2PF
1PL1	2PO
1PL2	2PQ
1PO	
1PQ	

The concentration and purity of the extracted DNA were evaluated using the Nanodrop microvolume sample retention system (Thermo Fisher Scientific, Waltham, MA, USA).

V3–V4 region of 16S rRNA gene (amplified using the primers described in Illumina 16S protocol: # 15044223 Rev. B) and *ITS2*-rDNA fungal sub-region (amplified using the following primers: *ITS3* PCR Forward Primer 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-GCATCGATGAAGAACGCAGC-3' and *ITS4* Reverse Primer 5' GTCTCGTGGGCTCGGAG-ATGTGTATAAGAGACAGTCCTCCGCTTATTGATATGC-3') were subjected to amplicon library preparation (according to Illumina's instructions, 16S Metagenomic Sequencing Library Preparation, Part # 15044223 Rev. B).

Sequencing was performed by MiSeq (2x300 paired-end, 600-cycle) Illumina platform (Illumina, San Diego, CA, USA).

Data are available from NCBI under accession number PRJNA641785 with SRA datasets: ITS SUB7670266, 16S SUB7669523.

Bioinformatic Analyses

The marker data were analyzed using qiime2 [42] (<https://qiime2.org>), according to the standard pipelines [43]. Briefly, quality trimming and OTU-picking was done using DADA2 [44], representative sequences were aligned using mafft [45], uninformative positions were masked and a phylogenetic tree was built with fasttree [46]. Alpha diversity values and beta diversity (i.e., UniFrac distance) were calculated on rarefied samples. Assessment of significant variation of alpha diversity between categories was determined using the Kruskal–Wallis test. Beta diversity significance (among categories) test was calculated with PERMANOVA and Mantel test, respectively. Taxonomic assignment was given to representative sequences using the most updated version of the SILVA database (release 132) [47], or (for fungal data) the UNITE database [48]. The feature classifier was trained using the qiime2 classify-sklearn plugin on the database; the same plugin also classifies the reads in the real dataset. Graphics about taxonomic composition and multivariate analyses were done using Calypso [49].

3. Results and Discussion

Table 3 reports the values of DNA concentration and purity for the two protocols. For all double extracted samples, the DNA concentration obtained with Protocol 1 is higher compared to that yielded by Protocol 2. The purity of the extracted DNA was evaluated in term of the ratio of absorbance at 260 and 280 nm. The high impurity of some of the samples treated with Protocol 2 may probably indicate

that the kit’s reagents acted also on wood extractives. This hypothesis is confirmed by the dark color of some of the DNA suspensions.

Table 3. Values of DNA concentration and purity obtained for the two extraction protocols.

Protocol 1—Maxwell®RSC Plant DNA Kit			Protocol 2—Maxwell®RSC PureFood GMO and Authentication Kit		
Sample	DNA Concentration (ng/μl)	260/280 Ratio	Sample	DNA Concentration (ng/μl)	260/280 Ratio
1N1	33.5	1.73	2N1	13.2	1.38
1N4	49.8	1.29	2N4	8.5	1.63
1N9	19.5	1.36	2N9	7.3	2.56
1N39	45.7	1.20	2N39	5	3.34
1N88	52.9	1.1	2N88	4	22.78
1PF	233.6	0.98	2PF	123.3	1.05
1PO	67.3	1.27	2PO	40.1	40.1
1PQ	780.4	0.91	2PQ	35.9	35.9
1PL1	170.8	1.11			
1PL2	551.7	0.96			

The extracts obtained with both protocols were used for the preparation of the libraries to check if there were differences in the library amplification efficiency. All samples were able to generate libraries with a concentration of 4 nM and were loaded onto the flow cell. The raw data (Table S1) showed that all libraries yielded an adequate number of reads.

The Principal Component Analysis (PCA) calculated on the ecological matrix for 16S at the level of order, as expected, well separates N woods from the P ones (Figure 1), regardless of the extraction method.

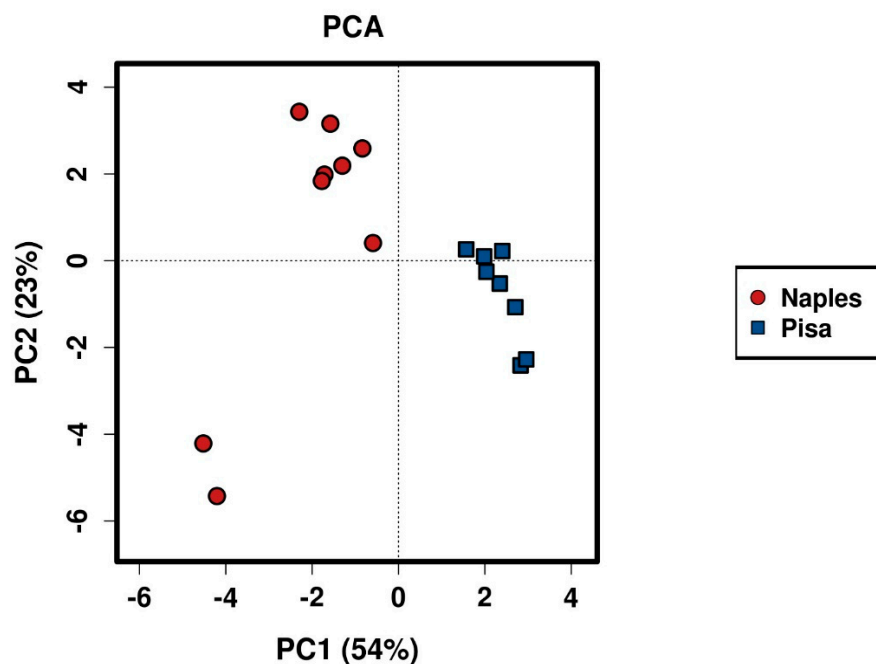


Figure 1. Principal Component Analysis calculated on the 16S ecological matrix at the level of order. The samples from Naples (red dots) and Pisa (blue square) show distinct clusters on the first axis that accounts for the 54% of the total variance.

3.1. Bacterial Community

The Beta diversity reported in Figure 2a shows the generic distribution of microbial community considering the difference in the bacterial communities present in Naples and Pisa samples. The graphic highlights that the samples cluster in two well-defined groups, each representing an archaeological site. Alpha diversity, based on observed OTUs and on Shannon index, indicates the richness and the biodiversity of each sample community. Figure 2b shows the boxplot of Shannon index in the two sites. The median Shannon values for the two communities are similar but the index varies in a wider range for Naples samples.

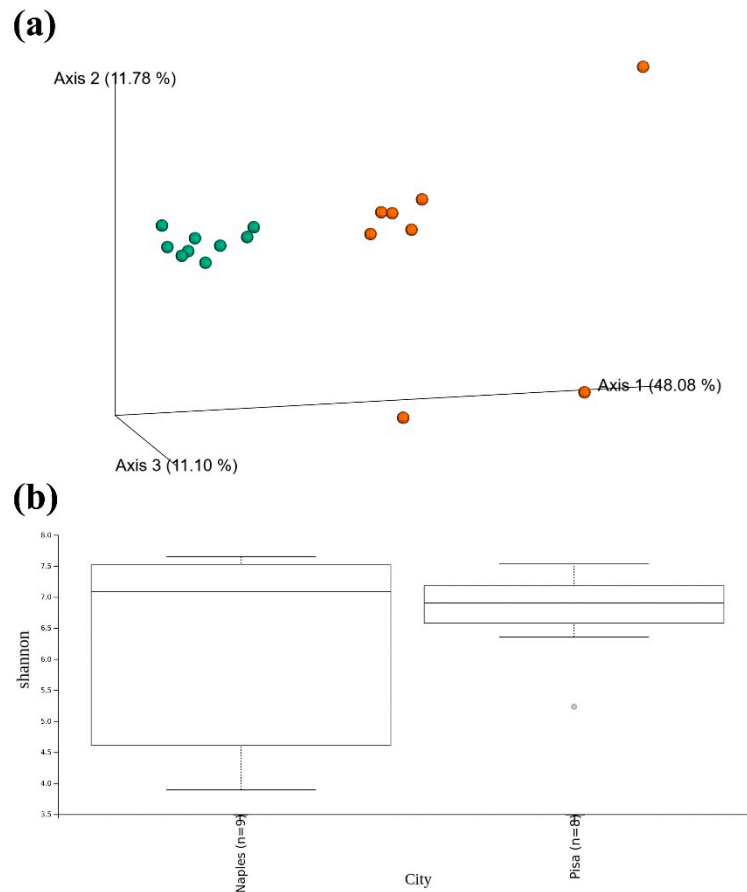


Figure 2. Diversity measures calculated on 16S rRNA gene sequencing data. (a) Beta diversity represented by Principle Coordinate Analysis Emperor plot on a Bray–Curtis distance matrix. Green dots: Naples, orange dots: Pisa. (b) Boxplots of Alpha-diversity Index (calculated as Shannon index) in the two archaeological sites.

Sequencing results showed that the bacterial communities of almost all the analyzed samples were dominated by taxa belonging to the phyla Proteobacteria, Acidobacteria, and Planctomycetes (Figure 3a). It is interesting to observe that the community composition obtained with the two extraction methods for the same wood sample is always very similar. This suggests that the results obtained are not biased by an extraction limit and that it can be considered as representative of the actual microbial community.

Proteobacteria accounted for more than 50% of the total reads in all the Naples samples, reaching 96% for *N88*, and more than 30% for Pisa woods. The phylum Bacteroidetes was well represented in Naples communities, in some cases reaching relative frequencies of 10–20%, while it accounted for less than 5% in Pisa samples. The opposite results were registered for the phylum Chloroflexi.

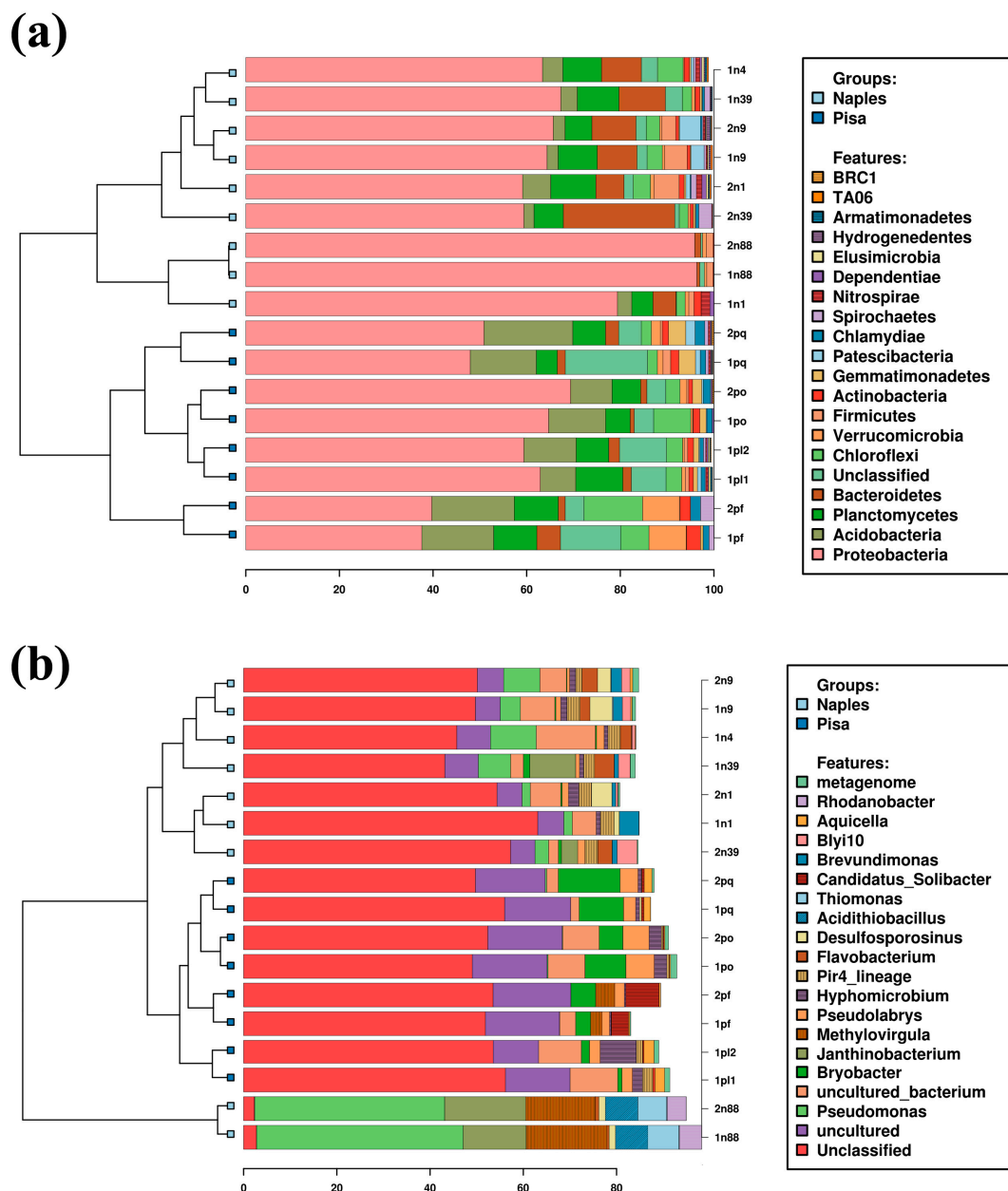


Figure 3. 16S. Stacked barcharts of the taxonomic profile at phylum (a) and genus (b) level.

At class level (Figure S1), the communities of N samples were mainly composed of Gammaproteobacteria, Alphaproteobacteria, Planctomycetacia, and Bacteroidia with a neat prevalence of the first taxon. Instead, Alphaproteobacteria dominated in P samples and together with Gammaproteobacteria, Planctomycetacia, and Acidobacteria accounted for more than 60% of the whole communities.

The communities of Naples samples were mainly composed of microorganisms belonging to the families Pseudomonadaceae (g. *Pseudomonas*), Burkholderiaceae (g. *Janthinobacterium*), Methylophilaceae, Pirellulaceae, and Xanthobacteraceae. Instead, the genera of the families Xanthobacteraceae (g. *Pseudolabrys*), Caulobacteraceae, Solibacteraceae (g. *Bryobacter*), and Hyphomicrobiaceae (g. *Hyphomicrobium*) were enriched in P samples (Figure S2 and Figure 3b).

Even if the role of bacteria in the biodegradation of WAW is well-known and several studies investigated the degradation patterns produced by these microorganisms [8,9,11,50,51], very little is known about the bacterial genera involved in this phenomenon. Some of the genera identified during

the present work (e.g., *Pseudomonas*, *Janthinobacterium*, *Flavobacterium*, *Brevundimonas*, *Sphingomonas*, and *Spirochaeta*) are known as members of the microbial community present in waterlogged wood and as active degraders of cellulose and/or lignin [22–25,29,31]. *Pseudomonas* sp. characterized the community of all the samples from Naples, reaching more than 40% of the relative frequency in N88, while the taxon was present only in four of the analyzed Pisa samples, always with frequencies lower than 0.5%. Microorganisms belonging to the genus *Pseudomonas* are strict aerobes or facultative anaerobes. Some species are involved in denitrification or are implicated in sulfur and iron metabolism [23]. A study carried out on chips of *Eucalyptus grandis* × *Eucalyptus urophylla*, *Populus canadensis*, and *Larix olgensis* demonstrated that the strain *Pseudomonas* sp. PKE117 is able to produce a wood weight loss ranging from 8% to 27% in 60 days. The characterization of the degraded wood showed that the lignin structure was degraded more than the cellulose [52]. Other studies demonstrated that several species belonging to the genus *Pseudomonas* are able to degrade lignin and lignin model compounds, to oxidize carbohydrates and to degrade cellulose via different metabolic pathways [53–59].

Species belonging to this genus are considered as cosmopolites and no specific relations have been reported with one or more wood species. In the present study, *Pseudomonas* sp. was identified in silver fir, elm, ash and holm oak samples. Landy et al. [23] reported *Pseudomonas* spp. from WAW pilings from different sites across Europe. The species were associated with spruce (*Picea abies* (L.) H.Karst.), fir, Scots pine (*Pinus sylvestris* L.), oak and poplar (*Populus* sp.). Palla et al. [25] identified *Pseudomonas* spp. from pine samples belonging to the wood recovered inside a rostrum in the site of Acqualadroni (Messina, Sicily). Wagner et al. [29] identified three *Pseudomonas* strains from *Quercus robur/petraea* remains recovered from different sites across Europe.

In WAW, the genus *Janthinobacterium* is reported in association with *Pseudomonas*, in samples coming from sites characterized by limy soil [23,29]. Some species of the genus are reported from lake sediments, as part of the bacterioplankton of maritime Antarctic lake and from Antarctic snow [60–62]. In the present study, it was identified only in N88 and N39, respectively silver fir and elm, in association with *Pseudomonas*. Ravindran and Yang [63] demonstrated that the strain *Janthinobacterium* sp. AR-129 has cellulolytic activity and is able to produce high thermal stable cellulase.

Flavobacterium was identified in all the Naples samples but not in Pisa. The genus is reported as part of the bacterial community of WAW piles coming from two archaeological sites from the Netherlands [23] and it has also been identified in the storage water of lacquerware from the Nanhai No. 1 shipwreck (China) [31]. Members of the genus *Flavobacterium* have a wide distribution, they mostly occur in aquatic ecosystems ranging in salinity from freshwater to seawater, but have also been isolated from soil and sediments [64]. The species *F. akiainvivens* was isolated from decaying wood of the Hawaiian shrub *Wikstroemia oahuensis* [65]. The known species belonging to this genus are able to degrade cellulose derivatives but not crystalline cellulose, some strains have proved to be able to lysate algae [64,66].

Sphingomonas was found in samples N4, N9 and N39. It was isolated from spruce, Scots pine, and oak pile dwellings and from the Aqualadroni rostrum [23,25]. The species belonging to this genus are usually isolated from soil but can also be animal and human pathogens [67–70]. *Sphingomonas* strains are able to degrade cellulose, lignin and lignin derivatives [57–59,71].

Some of the identified genera never reported before in WAW deserve a special mention. The genus *Methylovirgula* was identified only in two samples N88 (silver fir) and PF (ash). It is part of the bacterial community present in decaying wood of the species *Fagus sylvatica* (beech), *Picea abies* and *Pinus sylvestris* [72,73]. The species *Methylovirgula ligni* has been found on beechwood blocks attacked by white-rot fungi [74]. *Methylovirgula* bacteria are obligated methylotrophs, and can use methanol as the sole carbon source. Methanol is produced during the decomposition of woody materials and this could explain why these microorganisms are found in wood colonized by fungi and in advanced stages of decay [72,73].

The genus *Bryobacter* was identified in both Naples and Pisa samples. In the latter, it reached a relative frequency of 13%, while in N wood the frequency was always equal to or lower

than 1%. The genus comprises acidotolerant, strictly aerobic, slow-growing chemoorganotrophic bacteria, which inhabit acidic wetlands and soils and are capable of hydrolyzing several heteropolysaccharides [75]. Currently, only the species *Bryobacter aggregatus* is described for this genus, it was isolated from boreal Sphagnum peat bogs. This species is able to hydrolyze several substrates, among which pectin and starch, but not cellulose [76].

Most of the other identified genera are usually isolated from soil and sediments or from water (freshwater or seawater) (e.g., *Pseudolabrys*, *Hyphomicrobium*, *Desulfosporosinus*, *Rhodanobacter*, *Aquicella*, *Reyranella*, *Devosia*, *Dongia*, and *Curvibacter*) [77–88]. Species belonging to some of the identified genera are reported as sulfur and iron-oxidizing (e.g., *Acidithiobacillus*, *Thiomonas*, *Pseudolabrys*, and *Hyphomicrobium*) [89–93] or denitrifying bacteria (e.g. *Rhodanobacter*) [94,95]. It is interesting to note that the genera *Acidithiobacillus*, *Thiomonas* and *Rhodanobacter* were identified only in sample N88, indicating a concentration of sulfur, iron and nitrogen in the wood. SEM-EDS analyses carried out on the sample during an experimentation external to the present work confirmed the presence of sulfur and iron (iron aluminosilicate) in the wood (data not published).

3.2. Fungal Community

As for the 16S data, the Beta diversity index (Figure 4a) shows a difference between the fungal communities of Naples and Pisa samples. The median Shannon values, indicating Alpha diversity, are similar for the two communities (Figure 4b).

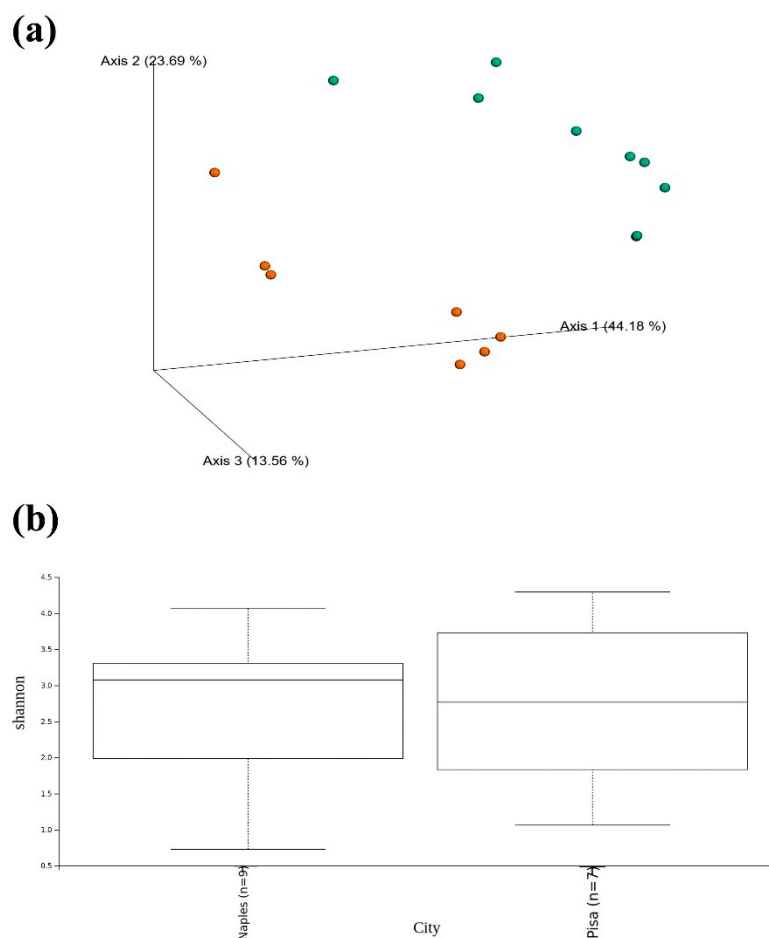


Figure 4. Diversity measures of ITS2 sequencing data. (a) Beta diversity represented by Principle Coordinate Analysis Emperor plot on a Bray–Curtis distance matrix. Green dots: Naples, orange dots: Pisa. (b) Boxplots of Alpha-diversity Index (calculated as Shannon index) in the two archaeological sites.

The sequencing results showed that the fungal communities of the wood samples coming from Naples were dominated by the phylum Ascomycota, accounting on average for more than 50% and reaching in some cases more than 90% of the total reads (Figure 5a). In Pisa samples, a neat prevalence of the phylum Basidiomycota was registered. Sequences attributed to this phylum represented more than 65% of the identified taxa in almost all analyzed samples. It is interesting to note the presence of sequences belonging to the fungal phyla Chytridiomycota and Rozellomycota in some of the P samples and of the phylum Cercozoa (supergroup Rhizaria) in both N and P samples (reaching more than 67% in sample PO). Again, the extraction method does not seem to have modulated the composition of the fungal community (Figure 5a).

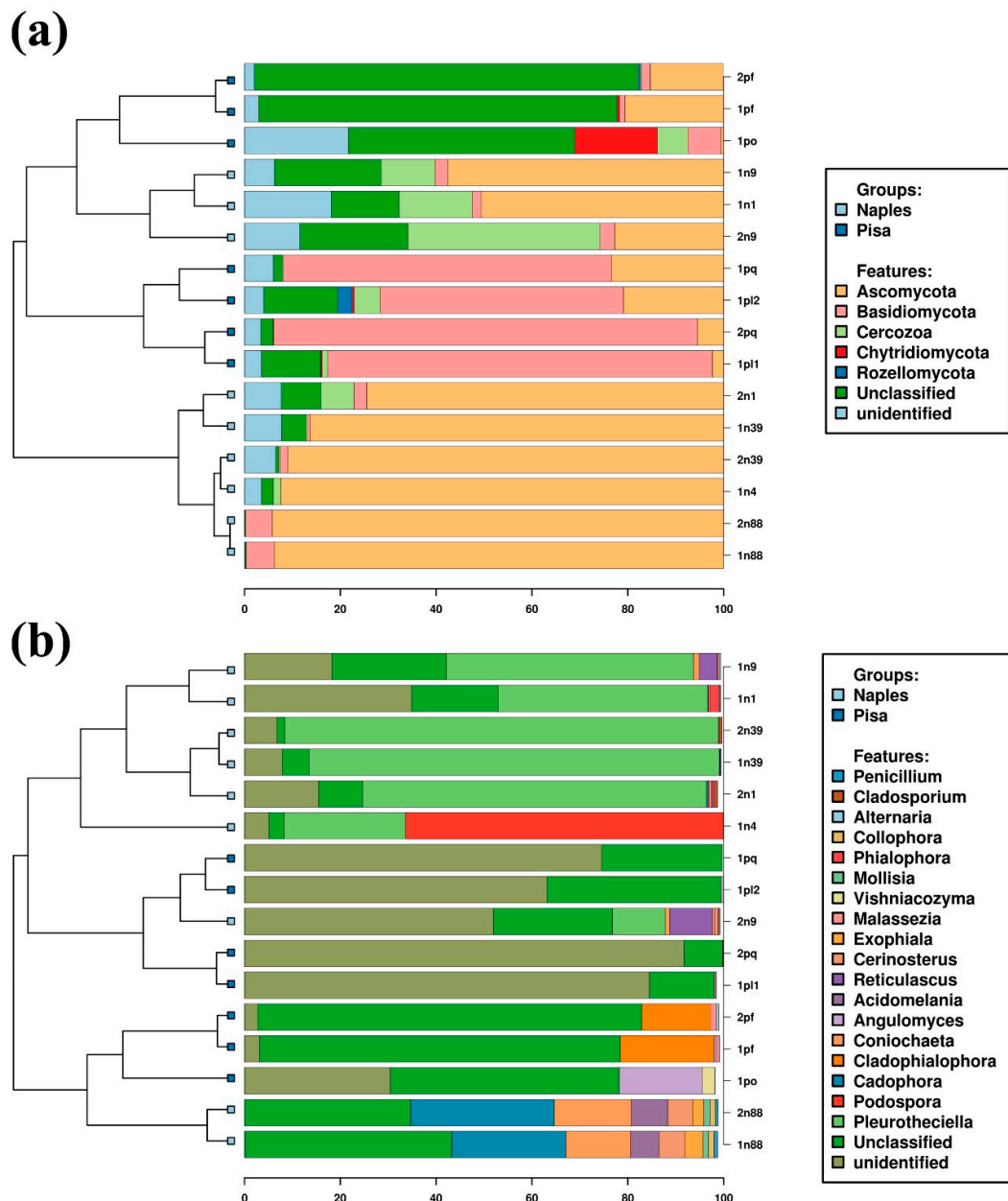


Figure 5. ITS2. Stacked barcharts of the taxonomic profile at phylum (a) and genus (b) level.

At class level (Figure S3), the communities of N samples were mainly composed of Sordariomycetes (on average 55% of the total reads), Leotiomyces (on average 39%), and Dacrymycetes (5% of the N88 community). Agaricomycetes dominated the P samples, accounting for more than 70% of the total reads on average. The class Dothideomycetes was identified in almost all analyzed samples and reached more than 5% of the relative frequency in most of them. The class Eurotiomycetes was present only in sample PF accounting for 14% (Protocol 2) and 19% (Protocol 1) of total reads.

The most represented families in N samples are Pleurotheciaceae (genus *Pleurotheciella*), Lasiosphaeriaceae (g. *Podospora*), Coniochaetaceae (g. *Coniochaeta*), Herpotrichiellaceae (g. *Coniosporium*, *Phialophora* and *Exophiala*), Dacrymycetaceae (g. *Cerinosterus*, *Calocera*) and Reticulascaceae (g. *Reticulascus*). Pleurotheciaceae accounted for more than 50% of total reads in all analyzed samples except for N88. The genera of the families Serendipitaceae (unidentified genus) and Herpotrichiellaceae (g. *Cladophialophora*), were enriched in P samples. In particular, the family Serendipitaceae accounted for a percentage of reads ranging from 2% to 88% in the different samples (Figure S4 and Figure 5b).

Differently from what has been observed in the bacterial communities, most of fungal genera were exclusively identified in one or two of the analyzed samples (Figure 5b). Usually, the relative frequencies obtained for each taxon with the two extraction protocols do not coincide; therefore, in the results, both values are reported.

Fungal identification at species level through ITS barcoding is not very reliable due to problems linked to the insufficient hypervariability and/or amplicon length (especially for species-rich ascomycete genera), insufficient annotations in public DNA repositories, and the unreliable sequences deposited in the reference databases [96–98]. However, in the discussion of the results some of the identified species (Figure S5) will be mentioned due to the interest they have for the present work.

The results obtained for Naples samples will be analyzed first. *Pleurotheciella* is the most abundant genus in all analyzed N samples except for N88 where it is absent. Species of the genus *Pleurotheciella* are usually isolated from freshwater habitats, the species *P. rivularia* (identified in the N samples) was collected on decaying wood submerged in freshwater [99,100]. The literature has not reported a possible ligninolytic or cellulolytic activity of this species.

The genus *Cadophora* was identified in samples N88 (relative frequencies 23%—Protocol 1 and 29.9%—Protocol 2), N39 (0.1%—Protocol 1, not detected with Protocol 2) and N1 (0.3%—Protocol 1, not detected with Protocol 2). Species belonging to the genus *Cadophora* are reported from decaying wood, soil and plants [101,102], from wooden artifacts from the Ross Sea region (Antarctica) and Deception Island (South Shetlands, Antarctica) and from decayed arctic driftwoods [103–106]. *C. malorum*, *C. luteo-olivacea*, *C. fastigiata* and previously undescribed *Cadophora* species designated as *C. species H*, *C. species E* and *C. species NH* were proved to cause type 1 soft rot after 12 months of incubation on *Betula* and *Populus* sound wood [103].

The genus *Podospora* accounted for more than 66% of total reads of the fungal community of sample N4. *Podospora* species are saprophytic, predominantly reported as coprophilous [107–110]. *Acidomelania* (species *A. panicicola*, 5.9%—Protocol 1 and 7.6%—Protocol 2) and *Cerinosterus* (species *C. luteoalbus*, frequencies 5.3%—Protocol 1 and 5.2%—Protocol 2) were found only in sample N88. The first species is closely related to endophyte species and is usually isolated from the roots of plants living in acid and nutrient-poor environments [111]. The latter was isolated from decayed historic wood on Deception Island (South Shetlands, Antarctica) and from arctic driftwood attacked by soft-rot fungi [105,106].

Some of the less abundant taxa deserve a special mention. *Reticulascus clavatus* was exclusively found in sample N9 (3.7%—Protocol 1 and 9%—Protocol 2). It is reported as the teleomorph form of *Cylindrotrichum clavatum*. The species was isolated from submerged wood of *Alnus glutinosa*, *Platanus* sp., *Fraxinus* sp. and from decayed wood of *Ulmus scabra* [112]. *Mollisia dextrinospora*, found in sample N88 (1.1%—Protocol 1 and 1.3%—Protocol 2) is reported as the teleomorph form of a *Cadophora*-like anamorph [113]. It was isolated from decaying wood [114]. *Lecythophora (Coniochaeta) hoffmannii*, found only in sample N9 (1.2%—Protocol 1 and 0.8%—Protocol 2), is a facultative plant

pathogen; it can colonize as saprotroph soils, leaf litter, and coarse wood debris or cause opportunistic mycosis in humans. Nevertheless, it is also reported as a soft-rot fungi, able to degrade the aromatic compounds (phenolics and aryl alcohols/aldehydes) that are produced during wood decomposition thanks to a pool of extracellular enzymes [115,116]. The genus *Penicillium* includes species reported as soil inhabitants able to degrade lignin, lignin-related aromatic compounds, and cellulose or as soft-rot agents [117–121]. *Penicillium simplicissimum*, identified in sample N88 (0.7%—Protocol 1 and 0.5%—Protocol 2), is not considered a white-rot fungus but several works demonstrated its ability to degrade lignin and lignin-related compounds derivate from lignin degradation by wood-rot fungi [122–125]. *Exophiala* was part of the fungal communities of samples N9 (0.1%—Protocol 1 and 0.4%—Protocol 2) and N88 (3.7%—Protocol 1 and 2.2%—Protocol 2). The genus includes agents of opportunistic infection in humans, potentially able to cause a wide diversity of mycoses, varying from cutaneous infections to disseminated syndromes [126–128]. Moreover, *E. xenobiotica* is reported as an opportunistic black yeast isolated from hydrocarbon-rich environments [129].

The fungal communities of Pisa samples are more difficult to interpret. Most of the obtained sequences remained unidentified; for some samples, more than 60% of total reads are generically reported as Fungi. However, in four of the analyzed woods, more than 71% of sequences are reported as belonging to the family Serendipitaceae (Figure S4), therefore these data suggest that the fungal communities of the Pisa samples are dominated by basidiomycetes. Several fungal species belonging to the phylum Basidiomycota are well known wood degraders. A large number of basidiomycetes have been typified as brown or white rot fungi, able to degrade cellulose and lignin [5,130–134]. Among the few identified species, the most abundant basidiomycetes are *Angulomyces argentinensis* and *Schizophyllum commune*. The first was identified in samples PO (17.2%—Protocol 1 and 0.9%—Protocol 2), PF (0.4%—Protocol 1, not detected with Protocol 2), PL1 (relative frequency 0.2%), and PL2 (relative frequency 0.4%). It has been isolated from water, pollen, soil and cellulosic material in tropical ponds [135,136]. *S. commune*, found only in sample PO (1.6%—Protocol 1, not detected with Protocol 2), is one of the most widely distributed white-rot fungi. Several studies proved its ability to degrade cellulose and lignin through different enzymatic processes [137–143].

Among the ascomycetes identified from Pisa samples, the genus *Cladophialophora*, found in sample PF (19.5%—Protocol 1 and 14.3%—Protocol 2), deserves a mention. Species belonging to this genus have been reported as degrader of wooden artifacts from the Deception Island (South Shetlands, Antarctica) [105]. The species *C. bantiana*, identified in this study, is a thermotolerant multinucleated saprophytic black mold, isolated from decayed vegetation, wood and soil able to cause myelitis in humans [144,145].

3.3. Microbiota and Wood Decay

For both *16S* and *ITS2* sequences, it was observed that the communities of the samples treated according to the two extraction protocols were very similar, as it can be clearly observed in Figures 3 and 5. This suggests that the obtained results can be considered as representative of the actual communities present in the wood. The differences in the relative frequency registered for almost all identified taxa could be attributed to the extraction method or to the anisotropic distribution of the microorganisms inside the wood.

On the contrary, the neat differences observed in the composition of microbial communities in Naples and Pisa samples should be attributed to the lying site. In fact, it is well known that the heterogeneous environmental conditions of the soil (e.g., pH, climate, organic carbon availability) influence the microbial community [146,147], and soil represents the main source of colonizers for wood in ground contact [148,149]. This statement is obviously valid also for WAW that lies at a certain depth included in waterlogged soil for centuries and that, during excavation and recovery, comes into contact with the most superficial ground layers, the richest in term of microbial biomass and diversity.

By analyzing more in depth the differences among the bacterial communities of the two sites, it is evident that Naples samples were characterized by the highest number of potential biodeteriogen

bacterial genera (*Pseudomonas*, *Janthinobacterium*, *Flavobacterium*, *Sphingomonas*, *Methylovirgula*, *Bryobacter*, *Acidithiobacillus*, *Thiomonas*, and *Rhodanobacter*) whereas only three of these taxa, *Pseudomonas*, *Methylovirgula*, and *Bryobacter* were present in Pisa woods. Regarding the fungal communities, 10 of the 13 potential biodeteriogen taxa identified were present only in Naples samples (*Pleurotheciella*, *Cadophora*, *Podospora*, *Acidomelania*, *Cerinosterus*, *Reticulascus*, *Mollisia*, *Lecythophora*, *Penicillium*, and *Exophiala*) while three were exclusive of Pisa (*Angulomyces argentinensis*, *Schizophyllum commune*, *Cladophialophora*). In this case, it worth remembering that lot of the sequences extracted from Pisa samples remained unidentified so the presence of other ligninolytic and/or cellulolytic species cannot be excluded.

Table 4 reports the results of the physical analyses carried out to evaluate the level of degradation of Pisa woods in 2008 and 2018. Data clearly show a general increase in the degradation of wood. For holm oak and oak, an increase by 100–200% of MWC and by 4% of LWS values was registered while for elm and ash MWC almost doubled and LWS increased by ca 5–10%. Degradation occurring during the 10 years of storage could be attributed to the ongoing activity of biodeteriogens present inside the wood, particularly to the fungi (mostly basidiomycetes) which are better competitors with respect to bacteria. It is worth underlining that basidiomycetes proved to have a high degradative potential even in condition of complete imbibition, usually considered as protective for WAW against these biodeteriogens.

Table 4. Results of physical characterization of Pisa wood samples. Data of 2008 analyses from: Cragnolini, E.; Sarra, A. Sperimentazione di metodi di impregnazione e liofilizzazione di legni archeologici provenienti da ambiente saturo d'acqua, Istituto Centrale per il Restauro, 2008.

	Sample	D _{bd} (g × cm ⁻³)	MWC (%)	LWS (%)
2008	Elm 1	0.20	441	63
	Elm 2	0.18	517	67
	Holm oak	0.16	582	78
	Oak	0.20	453	71
	Ash	0.14	657	78
2018	PF	0.09	1102	83
	PO	0.11	879	81
	PQ	0.17	530	75
	PL	0.13	729	82

Finally, it is interesting to note that the bacterial genus *Methylovirgula* was present only in sample PF. As discussed, these bacteria are obligated to use methanol as carbon source. As this molecule is a degradation product of woody materials, it is probable that it was particularly concentrated in PF which was characterized by an advanced state of decay.

4. Conclusions

As the discussed results demonstrate, much of the identified bacterial and fungal taxa have cellulolytic or ligninolytic abilities. The potential biodeteriogens were present in the samples from both archaeological sites and no correlation emerged between their presence and the wood species and/or the storage conditions. This means that all WAW recovered from the sites should be considered at risk of biological degradation during the storage pre-restoration phase. The results of physical analyses carried out on wood stored at room temperature showed that a neat increase of the level of degradation can be registered over a 10-year period. It can be supposed that, in the wood preserved at 4 °C, the microorganisms' activity is reduced but it cannot be completely excluded.

Hence, the results obtained in the present work should increase the awareness of conservators and restorers on the importance of adopting suitable practices for the prevention of biodeterioration. The supplement to the Official Gazette of the Italian Republic n. 244 (2001), also known as “Museum Standards”, prescribes the use of a biocide during storage and restoration phases, but when it is not possible to attend this prescription, alternative strategies should be taken into account (e.g., change the storage water frequently, use of UV light to sterilize water, shorten storage and restoration time). Finally, the use of high-throughput sequencing analyses during these tricky periods could help in defining the complexity of the microbiota present in WAW highlighting the presence of possibly biodeteriogen taxa.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2076-3417/10/13/4636/s1>. Table S1: Raw data obtained for 16S and ITS with the two extraction protocols. Figure S1: 16S. Stacked barcharts of the taxonomic profile at class level, Figure S2: 16S. Stacked barcharts of the taxonomic profile at family level, Figure S3: ITS2. Stacked barcharts of the taxonomic profile at class level, Figure S4: ITS2. Stacked barcharts of the taxonomic profile at family level, Figure S5: ITS2. Stacked barcharts of the taxonomic profile at species level.

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